IgG are dominated by those of its antigen-binding sites even after the specific binding of hapten or hapten-carrier at the combining sites. By contrast, the surface properties of protein–antibody complexes are not related to those of the unliganded antibodies. Instead, the surface properties of protein–IgG complexes are related mainly to those of the antigens. Depending on the type of antigen (hapten or protein), LLPC may thus be used to separate antigen–IgG complexes through differences in exposed surfaces of either the antibody combining sites or the antigen. LLPC may thus provide us with new, interesting information concerning the events upon complex formation in solution and possible ligand-induced conformational changes.

See also: **III/Nucleic Acids:** Liquid Chromatography. **Proteins:** Ion Exchange. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins. Essential Guides for Isolation/Purification of Immunoglobulins. Essential Guides for Isolation/Purification of Nucleic Acids.**

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Physico-Chemical Measurements

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Chromatography theory establishes that the retention volume of a solute is a function of its distribution coefficient and the variance of the eluted peak is a function of solute diffusivity. Consequently, retention volume measurements can provide distribution coefficient data which, if determined over a range of temperatures, will disclose the necessary thermodynamic information that will help reveal the physical nature of the distribution process. In addition, peak dispersion measurements can furnish accurate diffusion coefficients for almost any solute, in any liquid, at any chosen temperature, assuming appropriate solute solubility and temperature stability. Hence, the use of liquid chromatography to measure physicochemical properties of a distribution system hinges on the accurate measurement of retention volumes and peak widths.

Gas chromatography retention volume measurements have been used to provide a significant amount of thermodynamic data, including standard free energies, enthalpies and entropies of distribution, together with activity coefficients. These types of physicochemical measurements were pioneered in the late 1950s. In contrast, the use of liquid chromatography

for the same purposes has not been nearly so prevalent, although in many ways it can be a simpler and a more accurate experimental procedure.

Basic Thermodynamic Theory of Solute Distribution in Liquid Chromatography

The expression that relates the change in *standard free energy* of a solute when transferring from one phase to the other, as a function of the equilibrium constant (the distribution coefficient, K) is as follows:

$$RT \ln K = -\Delta G^0 \qquad [1]$$

where R is the gas constant, T is the absolute temperature and ΔG^0 is the standard free energy change.

Now:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \qquad [2]$$

where ΔH^0 is the standard enthalpy change and ΔS^0 is the standard entropy change.

The enthalpy term represents the energy involved when the solute molecule interacts by electrical forces with a molecule of the stationary phase. However, when interaction takes place, the freedom of movement of the solute molecule is also reduced and it can no longer move in the same random manner that it did in the mobile phase. This new motion restriction is measured as an entropy change. Thus, the free energy change is made up of an actual energy change which results from the work done during the interaction of the solute molecules with those of the stationary phase, and an entropy change that accounts for the resulting restricted movement or loss of randomness, when the solute is associating with the stationary phase.

Continuing:

$$\ln K = -\left(\frac{\Delta H^0}{RT} - \frac{\Delta S^0}{R}\right) \qquad [3]$$

It is seen that if the standard entropy change and standard enthalpy change for the distribution of any given solute between two phases could be calculated, then the distribution coefficient (K) and, consequently, its retention volume could also be predicted. Unfortunately, these properties of a distribution system are bulk properties that include, in a single measurement, the effect of all the different types of molecular interactions that are taking place between the solute and the two phases. As a result, it is often difficult to isolate the individual interactive contributions in order to estimate the magnitude of the overall distribution coefficient, or identify how it can be controlled. Nevertheless, there are a number of ways in which this can be done and, in any event, the thermodynamic approach can provide valuable information with regard to the nature of the distribution.

Rearranging eqn [3]

$$\ln K = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \qquad [4]$$

Bearing in mind:

$$V'_r = KV_s \qquad \text{and} \qquad k = \frac{KV_s}{V_m} = \frac{K}{a} \qquad [5]$$

where V'_r is the corrected retention volume of the solute, V_s is the volume of stationary phase in the column, V_m is the volume of mobile phase in the column, a is the phase ratio V_m/V_s, and k is the retention factor of the solute. Thus:

$$\ln(V'_r) = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln(V_s) \qquad [6]$$

or:

$$\ln(k) = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} - \ln(a) \qquad [7]$$

It is seen that a curve relating $\ln(V'_r)$ or $\ln(k)$ to $1/T$ should give a straight line, the slope of which will be proportional to the standard enthalpy change during solute transfer and the intercept will be related to the standard entropy change (but will include the logarithm of the phase ratio). Thus, the dominant thermodynamic contribution to any specific distribution system can be identified from such curves.

Such curves are called van't Hoff curves and an example of two exaggerated theoretical van't Hoff curves relating $\log(V'_r)$ against $1/T$ for two different types of distribution system is shown in **Figure 1**. It is seen that distribution system (A) has a large enthalpy value $[\Delta H^0/RT]_A$ and a low entropy contribution $[-(\Delta S^0/R) + V_S]_A$. The large value of $[\Delta H^0/RT]_A$ means that the distribution is predominantly controlled by molecular forces. The solute is preferentially distributed in the stationary phase as a result of the interactions of the solute molecules with those of the stationary phase being much greater than the interactive forces between the solute molecules and those of the mobile phase. Because the change in enthalpy is the major contribution to the change in free energy, the distribution, in thermodynamic terms, is said to be energy driven.

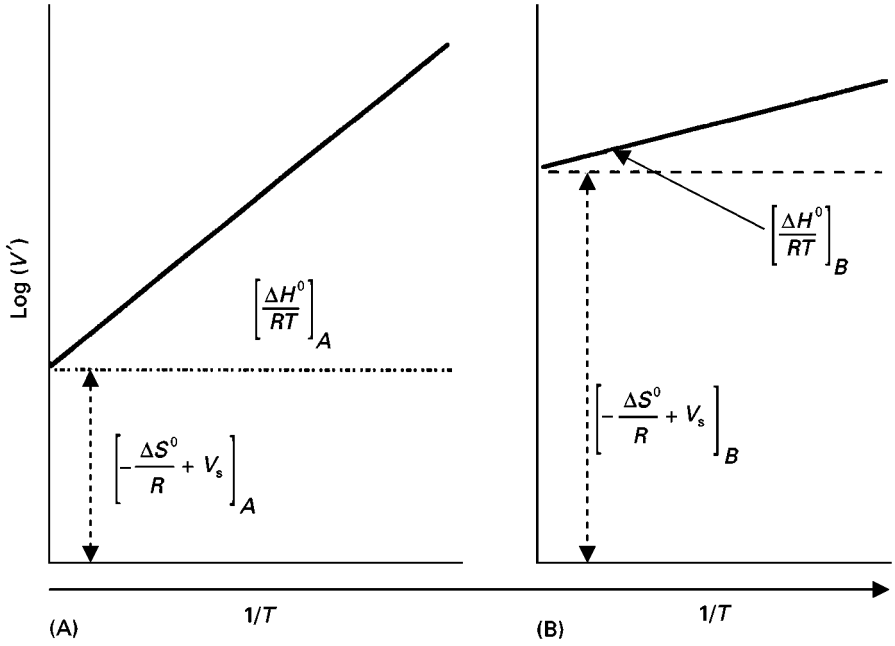

Figure 1 Van't Hoff curves for two different distribution systems. (A) Energy-driven distribution; (B) entropically driven distribution.

In contrast, it is seen that for distribution system B there is only a small enthalpy change $[\Delta H^0/RT]_A$, but in this case a high entropy contribution $[-(\Delta S^0/R) + V_S]_B$. This means that the distribution is not predominantly controlled by molecular forces. The entropy change reflects the loss of randomness or freedom that a solute molecule possessed when transferring from one phase to the other. The more random and more free the solute molecule is to move in a particular phase, the greater is its entropy in that phase. In system B, the large entropy change indicates that the solute molecules are much more restricted or their movements less random in the stationary phase than they were in the mobile phase. Because the change in entropy in system B is the major contribution to the change in free energy, the distribution, in thermodynamic terms, is said to be entropically driven.

Chiral separations and separations dominated by size exclusion are examples where retention and/or selectivity would be entropically controlled. However, chromatographic separations cannot be exclusively energetically driven or entropically driven, but must contain both constituents. In some cases, by careful adjustment of both energetic and entropic components, very difficult and subtle separations can be accomplished.

In most distribution systems met in chromatography, the slope of the van't Hoff curves are positive and the intercept negative. The negative value of the intercept means that the standard entropy change of the solute has resulted from the production of a less random and more orderly system during the process of distribution into the stationary phase. More importantly, this entropy change reduces the magnitude of the distribution coefficient and attenuates solute retention. Conversely, the greater the forces between the molecules, the greater the energy (enthalpy) contribution, which increases the distribution coefficient and extends the retention. Thus, in liquid chromatography, the enthalpy and entropy changes tend to oppose one another in their effect on solute retention. In fact, there is considerable parallelism shown between the standard entropy and standard enthalpy of a series of solutes for a given distribution system.

This relationship between entropy and enthalpy has been reported many times in the literature. An example of a graph relating ΔH to ΔS is shown in **Figure 2**. Any increase in enthalpy indicates that more energy is used up in the association of the solute molecule with the molecules of the stationary phase. This means that the intermolecular forces are stronger and thus the stationary-phase molecules hold the solute molecules more tightly. In turn, this implies that the freedom of movement, and the random nature of the solute molecule, are also more restricted, which results in a larger change in standard entropy. It follows that, unless other significant retentive factors are present, any increase in standard enthalpy is usually accompanied by a corresponding increase in standard entropy. If the molecules of one of a pair of closely eluting isomers suffer a greater reduction in

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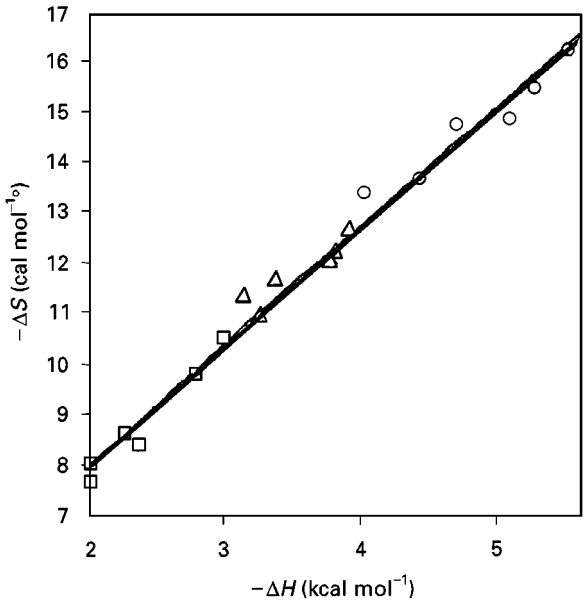

Figure 2 Graph plotting standard free entropy against standard free enthalpy for ether (triangles), thioether (squares) and amine (circles).

freedom or randomness relative to those of the other then, providing the energy changes are similar for the molecules of both isomers, those that experience the greater entropy change will be eluted first.

The van't Hoff curves can help provide even more information on the elution process. If the slope of the $\ln(k)$ versus $1/T$ curves (i.e. the standard free enthalpies) differ for two closely eluting isomers, then the lines must eventually intercept and there must be a temperature of co-elution and a subsequent elution order reversal. This elution order reversal is often not apparent in practice, as the co-elution temperature may occur outside the temperature range over which the distribution system will function satisfactorily for chromatographic purposes. However, in such distribution systems, which will include those used in most chiral separations, the operating temperature is of paramount importance in selecting the optimum operating conditions.

The Distribution of Standard Free Energy

The standard free energy can be used to explain chromatographic retention in two different ways. Firstly, portions of the standard free energy can be allotted to specific types of molecular interactions between the solute molecules and the two phases. Secondly, the molecule can be divided into different parts and each part allotted a portion of the standard free energy. This approach allows the contributions made by different parts of the molecule to retention to be explained. This concept was suggested by Martin in his early papers, and can be used to relate molecular structure to solute retention. An example of the second approach is as follows.

Thermodynamic Analysis of a Homologous Series

Consider the distribution of the standard free energy throughout an *n*-alkane molecule, allotting a portion to each methylene group and to the two methyl endgroups. Then, algebraically, this concept can be put in the following form:

$$RT\ln(V'_{r(T)}) = -n\Delta G_{(\text{methylene group})} - m\Delta G_{(\text{methyl group})} \quad [8]$$

where $\Delta G_{(\text{methylene group})}$ is the standard free energy of each methylene group, $\Delta G_{(\text{methyl group})}$ is the standard free energy of each methyl group, n is the number of methylene groups and m is the number of methyl groups ($m = 2$ for an *n*-alkane).

The concept will now be applied to a simple *n*-alkane series, the data for which was obtained on the stationary-phase *n*-heptadecane by Martire and his co-workers. This procedure will show how it is possible to identify the difference between the contribution of a methylene group and a methyl group to solute retention and how the difference can be explained. The curves for $\log(V'_{r(T)})$ against the number of methylene groups in each of the three *n*-alkanes for seven different temperatures are shown in **Figure 3**.

The expected straight lines are produced at each temperature and the indices of determination are very close to unity. The slope represents the portion of the standard free energy from each methylene group and each intercept represents the portion from the two methyl groups. It should be noted that the contribution of a single methyl group is seen to be significantly less than that from one methylene group. The slopes (the free energy contribution from a single methylene group, $\Delta G_{(\text{methylene group})}/RT$) and half the intercept (the contribution from one terminal methyl group $\Delta G_{(\text{methyl group})}/RT$) plotted against the reciprocal of the absolute temperature are shown in **Figure 4**.

These curves provide relative values for the standard enthalpy ($\Delta H_{(\text{methylene group})}/R$ and $\Delta H_{(\text{methyl group})}/R$) and standard entropy ($\Delta S_{(\text{methylene group})}/R$ and $\Delta S_{(\text{methyl group})}/R$) of the distribution for each group, the magnitude of which indicates the manner in which they interact with the stationary phase.

Despite the contribution of a methyl group to the free energy being much less than that of the methylene group, the energies of interaction (the enthalpies)

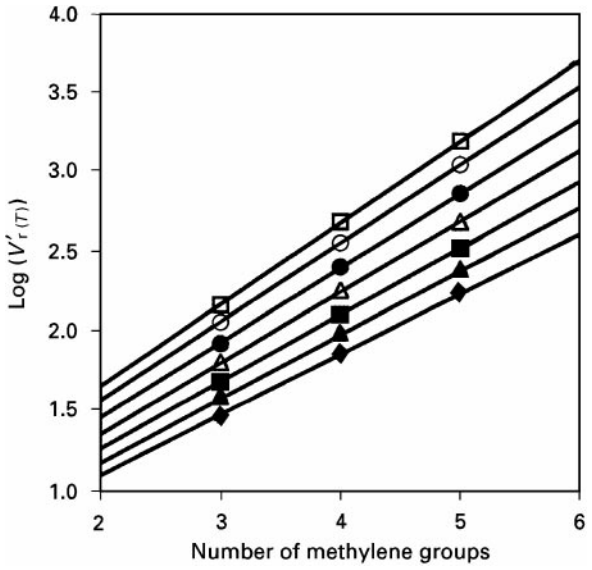

Figure 3 Graph showing log($V'_{r(T)}$) against number of methylene groups for a series of *n*-alkanes. □, 22.5°C; $y=0.5150x+0.6211$, $r=1.0000$. ○, 30°C; $y=0.4949x+0.5737$, $r=1.0000$. ●, 40°C; $y=0.4706x+0.5126$, $r=1.0000$. △, 50°C; $y=0.4448x+0.4631$, $r=1.0000$. ■, 60°C; $y=0.4216x+0.4196$, $r=1.0000$. ▲, 70°C; $y=0.4008x+0.3751$, $r=1.0000$. ◇, 80°C; $y=0.3792x+0.3399$, $r=1.0000$.

of the two groups are very similar. However, the entropy term for the methyl group is nearly 50% greater than that of the methylene group and, as this acts in opposition to the standard enthalpy contribution, it reduces the free energy associated with the methyl group by about 30% relative to that of the methylene group. This entropy difference between the two groups is due to the methylene group being situated in a chain (more rigidly held) and has, initially, a much lower entropy before solution in the stationary phase. In contrast, the methyl group, situated at the end of the chain, is much less restricted and thus, on interaction with the stationary phase molecules, the entropy change is much greater. It follows that the introduction of a methylene group into a solute molecule will increase its retention more than the introduction of a methyl group due to the greater change in entropy associated with the methyl group.

Thus, if different portions of the standard free energy of distribution are allotted to different parts of a molecule, then their contribution to solute retention can be revealed. Furthermore, from the relative values of the standard enthalpy and standard entropy of each portion or group, the manner in which the different groups interact with the stationary phase may also be revealed.

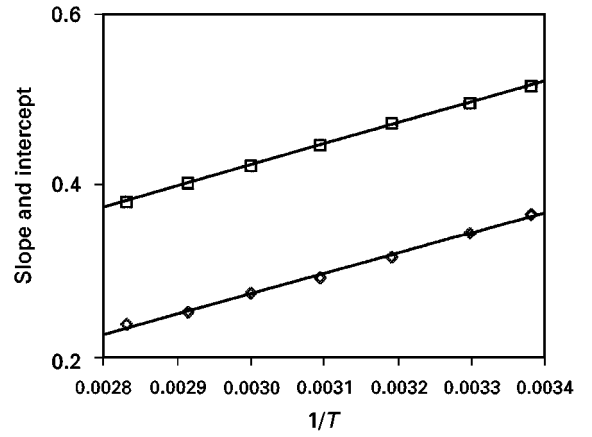

Figure 4 Graph showing intercept and slope from [log($V'_{r(T)}$)/number of CH_2 group curves] for a series of *n*-alkanes against the reciprocal of the absolute temperature. Squares, CH_2: $y=246.7281x-0.3186$; $r=0.9999$. Diamonds, CH_3 groups: $y=235.2839x-0.4333$; $r=0.9985$.

The Distribution of Standard Free Energy Between Different Types of Molecular Interactions

In contrast to apportioning the standard free energy between different groups in the solute molecule, it can also be divided between the different types of forces involved in the solute/phase–phase distribution. This approach has been elegantly developed by Martire in his unified theory of retention. In a simplified form, the standard free energy can be divided into portions that result from the different types of interaction, e.g.:

$$\Delta G = \Delta_{\text{dispersive}} + \Delta_{\text{polar}} + \Delta_{\text{ionic}} \qquad [9]$$

The polar interactions are often divided into weak, moderate and strong interactions that have, somewhat arbitrarily, been given terms such as π–π interactions, dipole–dipole interactions and hydrogen bonding:

$$\Delta G = \Delta_{\text{D}} + \Delta_{\pi-\pi\ \text{interactions}} + \Delta_{\text{dipole–dipole interactions}} + \Delta_{\text{hydrogen bonding}} \qquad [10]$$

Nevertheless, it is important to realize that they are not different types of interaction, but are all polar interactions, electrical in nature but of different strengths. In fact, many more terms have been introduced to describe subtly different enthalpic and entropic contributions to retention. In some cases, these extra terms are often introduced to take into account many second-order effects. It must be emphasized, however, that standard free energy is a bulk property, and where a portion is allotted to a particular type of interaction, e.g. dispersive, it will include all

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dispersive interactions throughout the whole molecule and not one specific interaction that arises from a particular chemical group.

This approach has been used to determine the so-called binding constants between solute and stationary phase and also to investigate complexation constants. Binding constants and complexation constants have ben introduced to distribution system theory largely to account for the wide range of strengths associated with polar interactions between molecules. It follows that some discussion of the physical nature of solute distribution between phases on the molecular level is necessary. Molecules can interact with one another by dispersive forces, polar forces and ionic forces, all of which are electrical in nature. Dispersive forces occur between randomly formed transient dipoles that are continuously generated throughout the molecule. For nonpolar substances, dispersive forces are proportional to their molar volume and polarizability. Polar forces result from charge concentrations dispersed at different parts of a molecule (overall electrically neutral) and can vary widely in strength. At the extreme, the energies involved can approach that of a chemical bond, as in hydrogen bonding. Polar forces may or may not be related to the dipole moment of the substance as internal electrical compensation can take place. Consequently, as dipole moments are calculated from bulk measurements made by external electrical fields, the results are frequently not related directly to the polarity of the substance. For example, the magnitude of the apparent dipole moment of dioxane, as calculated from bulk measurements, is 0.45 Debyes. This compares with 1.15 Debyes for diethyl ether, which theoretically should be about half that of dioxane. Yet dioxane is a very polar substance, soluble in water, whereas ether is weakly polar and relatively insoluble in water. Ionic forces result from the net charges on the molecule and are not germane to this discussion.

Polar interactions can result between molecules with permanent dipoles (which are often very strong) and also between a molecule with a permanent dipole and one which is polarizable (which at the extreme can be very weak). When a polarizable molecule comes into close proximity with a molecule having a permanent dipole, the electric field from the dipole induces a counter dipole in the polarizable molecule. This induced dipole acts in the same manner as a permanent dipole and the polar forces between the two dipoles result in a polar interaction between the molecules. The part of the molecule that is negatively charged due to an asymmetric accumulation of electrons has been given the term an electron donor site, whereas the area of electron depletion in the molecules (that balances the site of electron accumulation) has been given the term electron acceptor site. More simply, these are basically negatively and positively charged sites on the molecule. Because polar interactions have such a range of strengths, certain ranges of polarity have been assumed to be due to certain types of electrical interaction that have been given the terms nonbonding lone pair, bonding π orbital, vacant orbital, antibonding π orbital, antibonding σ orbital, all of which are based on the accepted electron configuration of the molecule. In practice, however, whatever the cause, the strength of the interaction, and thus its effect on solute retention, is directly related to the intensity of the interacting charges. From the standing of separation technology, whether the introduction of these alternative terms helps to explain the separation process is a moot point, as, even in theory, in most cases the individual effects are extremely difficult to isolate completely from one another.

Martire *et al.* developed a theory assuming that polar interactions constituted the formation of a complex between the solute and the stationary phase with its own equilibrium constant. The complex is assumed to have no vapour pressure and thus remains stationary in the column until, as a result of the equilibrium kinetics, it dissociates again into the individual components. The uncomplexed solute molecule then continues to migrate along the column until complexing again takes place. Experimental data obtained to test the theory led to the conclusion that those solutes that are retained without complexing are retained solely by dispersive forces, whereas retention by polar interactions is the result of complexing. This is one approach to the explanation of retention by polar interactions, but the subject, at this time, remains controversial. Doubtless, complexation can take place, and probably does so in cases like olefin retention on silver nitrate-doped stationary phases. However, if dispersive interactions (electrical interactions between randomly generated dipoles) can cause solute retention without the need to invoke the concept of complexation, it is not clear why polar interactions (electrical interaction between permanent or induced dipoles) need to be considered differently.

Experimental Procedures

The basic apparatus that is recommended for measuring retention volumes in liquid chromatography is shown in **Figure 5**.

The equipment is standard, but the column oven should be capable of controlling the column temperature within $\pm 0.1^{\circ}$C. In addition, some provision should be made for pre-heating the mobile phase to

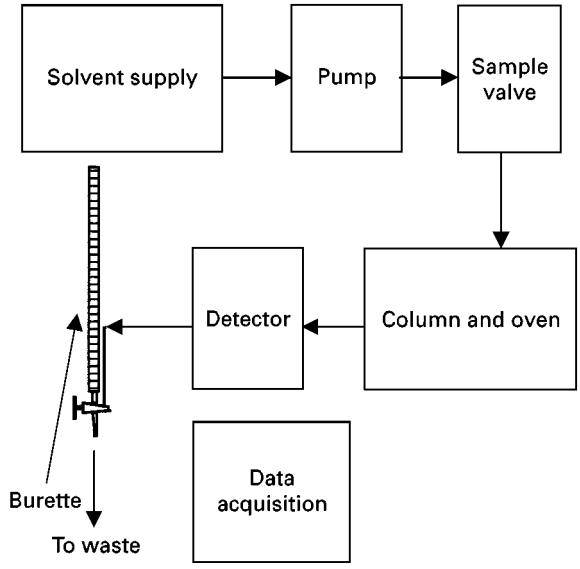

Figure 5 Apparatus for measuring retention volumes.

the correct temperature before it enters the column. The pressure drop across the column is expended as heat in the column, which can result in a significant rise in column temperature. To reduce this thermal effect, the pressure drop across the column must be made minimal and thus, very low flow rates should be employed. There are two basic methods used for measuring retention volumes. The first is to use a pump having a very constant flow rate and obtain values for the retention volume by multiplying the pump flow rate by the accurately measured retention time. The second, a better method, is to make absolute measurements of retention volume, using an accurately calibrated burette connected to the detector outlet.

This arrangement renders the use of an accurate and expensive pump unnecessary and eliminates any residual variation in pump flow rate that may still exist. The experimental procedure is as follows. The burette is read, the sample injected and the pump started. The detector output is observed and when a peak emerges, the pump is shut off at the peak maximum. The system is allowed a minute to reach equilibrium and the burette is read again. The difference between the two readings is taken as the retention volume being measured. Each retention volume should be measured in duplicate (preferably in triplicate) and any two measurements should not differ by more than 2%. After each retention volume measurement, the burette is drained to waste, until just above the lowest calibration, a few minutes allowed for drainage to be complete, and the next retention volume can then be measured. The retention volume is taken as the average of the replicates. The dead volume can be measured in a number of different ways and the numerous problems associated with dead volume measurement have been discussed in detail by Alhedai *et al.*

There are two column dead volumes that are used in liquid chromatography, the dynamic dead volume and the thermodynamic dead volume. The dynamic dead volume is the volume of the mobile phase that is actually moving, and is usually taken as the retention volume of an ionic salt such as sodium nitroprusside which, due to ionic exclusion, cannot enter the pores of the stationary phase or support. This value is used in peak dispersion theory calculations. The thermodynamic dead volume is taken as the total volume of mobile phase in the column. It is best obtained by weighing the column full of mobile phase, passing a gas through the column, and drying to constant weight. The difference between the full and empty column is taken as the thermodynamic dead volume. The retention volume of the solvent (or one component of a solvent mixture) carrying an isotopic atom can also be employed but a refractive index detector will probably be needed as the detector.

If the mobile phase consists of a mixture of solvents, then an approximate value for the thermodynamic dead volume can be taken as the retention volume of one pure component of the mixture. To minimize errors in dead volume measurement by this method, the retention factor (k) of any solute being examined should be in excess of 5. The corrected retention volume can be taken as the difference between the average retention volume of the solute and the dead volume. An example of a set of results relating corrected retention volume to the reciprocal of the absolute temperature is shown in **Figure 6**.

The information given in Figure 6 can be used in a number of ways. It is clear that enantiomer B has the higher standard free entropy and is thus the enantiomer that interacts more closely with the chiral stationary phase. The closer association of the B enantiomer with the stationary phase also results in a greater standard free enthalpy and the curves, V_r' against $1/T$, will thus have different slopes. Consequently, the two curves will intercept and, despite having different standard free entropies and enthalpies, there will be a temperature of co-elution above which the order of elution will be reversed. In this case, the elution order reversal was not observable as the co-elution temperature was above the maximum permissible for the particular stationary phase.

The linear equations resulting from a curve fit to the data will allow the retention factors (k) and the separation factor (α) of the two enantiomers to be calculated for any temperature. The analysis time, column length and minimum efficiency are a function

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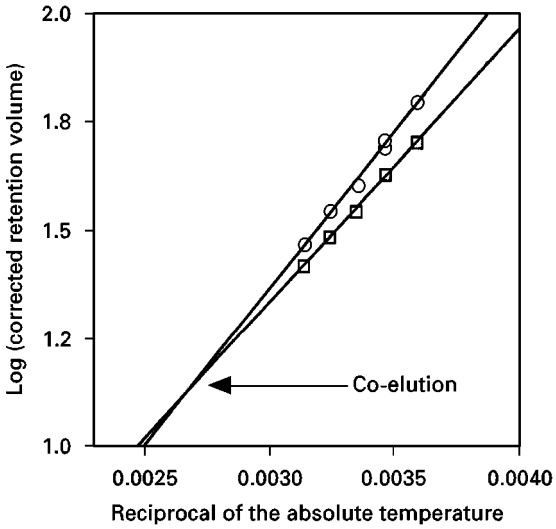

Figure 6 Graph plotting log (corrected retention volume) against the reciprocal of the absolute temperature for a pair of enantiomers separated on vancomycin. Squares, enantiomer A: $y = 631.1694x - 0.5614$; $r = 0.9992$. Circles, enantiomer B: $y = 725.8641x - 0.8116$; $r = 0.9993$.

of the retention factors and separation factor (α) of the two enantiomers. Thus, with an appropriate computer program, the optimum temperature, necessary efficiency and the optimum column length that will provide the minimum analysis time can be calculated from the data given in Figure 6. Finally, values for the standard free enthalpy and entropy for different solutes on different distribution systems can provide an insight into the nature and strength of the molecular interactions involved, which can then be related to molecular structure.

Chromatographic Methods for Measuring Diffusion Coefficients and Molecular Weights

The use of chromatography techniques to measure diffusion coefficients and molecular weights can be used very effectively where other techniques are difficult, cumbersome or time-consuming to employ. Diffusion coefficients are readily obtained by measuring the dispersion of a solute band in the liquid of interest as it passes through an open tube, but certain precautions must be taken. This technique follows directly from the theory of dispersion in open tubes developed by Golay. The van Deemter equation that describes dispersion occurring in a packed column can be used in a similar way to obtain an approximate estimation of the molecular weight of an eluted solute from a packed column.

Diffusion coefficients from dispersion measurements in open tubes Chromatographic techniques employing open tubes can be used very effectively to determine diffusion coefficients in liquids which, in turn, have been shown to be simply related to molecular weights. This work was pioneered in 1976 by Grushka and Kikta, who showed that the diffusion coefficient of a substance in a specific liquid (the diffusivity) could be determined by measuring its dispersion after passing through a cylindrical tube. According to the theory of band dispersion in an open cylindrical tube, as developed by Golay, the variance per unit length H of the solute band eluted from an open tube carrying no stationary phase is given by:

$$H = \frac{2D_m}{u} + \frac{r^2 u}{24D_m} \qquad [11]$$

where u is the linear velocity of the liquid through the tube, D_m is the diffusivity of the solute in the liquid and r is the radius of the tube.

Now, if $u \gg D_m/r$, then:

$$H = \frac{r^2 u}{24D_m} \qquad [12]$$

or:

$$D_m = \frac{r^2 u}{24H} \qquad [13]$$

As the value of D_m is generally less than 2.5×10^{-5} and the tube diameter is usually about 0.010 in i.d. then, $D_m/r < 10^{-3}\,\mathrm{cm\,s^{-1}}$, thus the condition $u \gg D_m/r$ is easily met in practice.

The apparatus is simple and consists of a solvent reservoir, a pump, a sample valve, the open tube and a suitable detector. The open tube should be jacketed by a larger tube containing circulating water from a thermostat. Temperature control should be $\pm 0.2^{\circ}$C. In order to ensure all measurements are accurate, however, some important conditions must be met. The sample valve must be an internal loop valve and have a capacity less than 0.2 μL; the detector cell must have a small volume certainly not greater than 5 μL and preferably less; the open tube must be connected directly to the detector cell with no intervening connecting tube (all commercial detectors have significant lengths of connecting tube between the column and the detector cell, and this connecting tube must be removed or bypassed so that the open tube is connected directly to the cell). These requirements are necessary to ensure that any extra tube dispersion is insignificant compared with that occurring in the open tube. In addition, solute diffusivity is pressure-dependent and thus low flow rates must be used to ensure a minimum pressure drop across the tube. Finally, there must be no radial flow introduced

in the open tube, as this aids diffusion and gives false high values. Consequently, the tube must be perfectly straight between the sample valve and the detector cell. Even a slight curve will produce radial flow and significantly increase the diffusivity as measured, and give false high values.

The band width is measured at the points of inflection at 0.6065 of the peak height. If manual measurements are used, the chart speed should be adjusted so that the peak width is at least 1 cm. The width should be measured with a comparator, reading to an accuracy of ± 0.1 mm. At least three replicate runs should be made and the three replicate values of efficiency should not differ by more than 2%. If the data acquisition system has software that will directly measure column efficiency, then this can be used providing its accuracy is carefully checked manually. Noise on the detector can often introduce inaccuracies that are less likely to occur with manual measurement.

Katz and Scott employed the technique to determine the diffusivities of 70 different solutes in an ethyl acetate–*n*-hexane solvent mixture. Using the data they also demonstrated a relatively simple relationship between the diffusivity and the molecular weight of the solute:

$$\frac{1}{D_m} = A + BV^{1/3}M^{1/2} = A + BM^{0.833}d^{-0.333} \quad [14]$$

where V is the molar volume of the solute, M is the molecular weight of the solute, A and B are constants for the solvent system employed, and d is the density of the solute.

Employing the values they obtained for the diffusivities of the different solutes, the relationship between diffusivity and molecular weight as given in eqn [14] is demonstrated by the curves relating $(1/D_m)$ against $(V^{1/3}M^{1/2})$, shown in **Figure 7**. It is clear that an approximate value of the molecular weight of an eluted solute can be obtained from the diffusivity of the solute which in turn will be related to the band width or the dispersion of the peak.

This procedure has been extended further by Katz to the measurement of high molecular weight materials such as polypeptides and proteins using a packed column. In the first instance, Katz found it necessary to identify the function of k which was included in the resistance to mass transfer term of the van Deemter equation. This was achieved by relating the difference between measured H values (the variance per unit length of the column) and the multipath term, to the reciprocal of the solute diffusivity, using solutes of known diffusivities. The system was then used to determine the molecular weight of a number of high molecular weight materials. The values obtained were not exceptionally accurate but were practically useful.

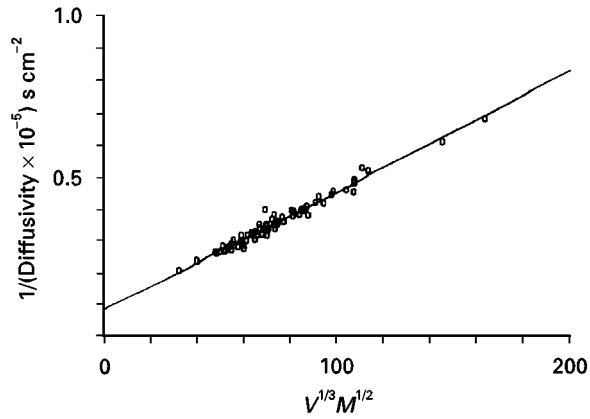

Figure 7 Graph relating the reciprocal of the diffusivity to the product of the cube root of the molar volume and the square root of the molecular weight. Courtesy of Elsevier Science.

In summary, the distribution coefficient and the thermodynamic properties of a distribution system can be easily measured by liquid chromatographic procedures. In addition, information can be gained about the nature of the distribution system and the molecular interactions involved. It is also possible to determine diffusion coefficients and approximate values for the molecular weights of eluted solutes by peak width measurements. Such data, however, may require some rather complex calibration procedures and the results, particularly with respect to the solute molecular weight, may have a precision of only $\pm 20\%$.

See also: **II/Chromatography: Liquid:** Theory of Liquid Chromatography. **III/Physico-Chemical Measurements: Gas Chromatography. Reverse-Flow Gas Chromatography.**

Further Reading

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Proteins

See **II / CHROMATOGRAPHY / Protein Separation**

Refractive Index Detectors in Liquid Chromatography

See **II / CHROMATOGRAPHY: LIQUID / Detectors: Refractive Index Detectors**

Size Exclusion Chromatography: Mechanisms

See **II / CHROMATOGRAPHY: LIQUID / Mechanisms: Size Exclusion Chromatography**

Theory of Liquid Chromatography

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Liquid chromatography (LC) involves the separation of the components of a mixture by virtue of differences in the equilibrium distribution of the components between two phases: one which is a liquid (the mobile phase) and moves in a definite direction, the other which is stationary (the stationary phase). Modern LC using very small particles and a relatively high inlet pressure is referred to as high performance liquid chromatography (HPLC).

Stationary Phase

An advantage of LC over gas chromatography is in the variety of types of stationary and mobile phases which may be used, allowing the separation of such diverse molecular species as pharmaceuticals, agrochemicals, proteins, inorganic and organic ions and biopolymers. The use of a stationary phase with a liquid coated physically onto an inert solid support (partition chromatography) has been superseded by the use of a liquid chemically bonded on to the solid support (bonded-phase chromatography). Ideally, the support does not contribute to the separation process, but its particle size and surface properties have a profound effect on the efficiency of the process.